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Effects of the SpoVT Regulatory Protein on the Germination and Germination Protein Levels of Spores of *Bacillus subtilis*

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Bacillus subtilis **isolates lacking the SpoVT protein, which regulates gene expression in developing forespores, gave spores that released their dipicolinic acid (DPA) via germinant receptor (GR)-dependent germination more rapidly than wild-type spores. Non-GR-dependent germination via dodecylamine was more rapid with *spoVT* spores, but germination via Ca-DPA was slower. The effects of a *spoVT* mutation on spore germination were seen with spores made in rich and poor media, and levels of SpoVT-LacZ were elevated 2-fold in poor-medium spores; however, elevated SpoVT levels were not the only cause of the slower GR-dependent germination of poor-medium spores. The *spoVT* spores had ≥5-fold higher GerA GR levels, ~2-fold elevated GerB GR levels, wild-type levels of a GerK GR subunit and the GerD protein required for normal GR-dependent germination, ~2.5-fold lower levels of the SpoVAD protein involved in DPA release in spore germination, and 30% lower levels of DNA protective α/β-type small, acid-soluble spore proteins. With one exception, the effects on protein levels in *spoVT* spores are consistent with the effects of SpoVT on forespore transcription. The *spoVT* spores were also more sensitive to UV radiation and outgrew slowly. While *spoVT* spores' elevated GR levels were consistent with their more rapid GR-dependent germination, detailed analysis of the results suggested that there is another gene product crucial for GR-dependent spore germination that is upregulated in the absence of SpoVT. Overall, these results indicate that SpoVT levels during spore formation have a major impact on the germination and the resistance of the resultant spores.**

Spores of *Bacillus* species are dormant and resistant to a variety of environmental stress factors and can remain in this state for years (35). However, spores constantly sense their environment, and if nutrients become available, spores can rapidly return to vegetative growth through the process of germination followed by outgrowth (25, 34, 35). Major spore proteins that sense nutrients are the germinant receptors (GRs), each of which senses a distinct germinant or mixture of germinants. *Bacillus subtilis* spores contain three major GRs: GerA, which alone triggers germination with L-valine or L-alanine, and GerB and GerK, which together trigger germination with a mixture of L-asparagine–D-glucose–D-fructose–K^+ (AGFK). A GerB variant in *B. subtilis* termed GerB* that can trigger spore germination with L-asparagine alone has also been isolated (23). The GRs are located in the spore's inner membrane, and each one contains three subunits, A, B, and C (5, 18, 19, 28, 34). The levels of these GRs are a major factor in determining rates of spore germination with particular germinants, and elevated GR levels lead to faster germination, while nutrient germination is essentially abolished in spores lacking GRs (4, 24). GR levels vary significantly between individual spores in populations, probably for stochastic reasons, since levels of GR subunits in spores are generally only 10s of molecules per spore (7, 8, 34). Rates of nutrient germination of spores can also vary significantly depending on the medium used for sporulation (6, 28, 29). In particular, spores made in poor media tend to germinate more poorly with nutrient germinants than spores made in a richer medium (10, 28). Recent work has further shown that with *B. subtilis* spores made in a poor medium, their slow germination with nutrients is paralleled by 2- to 5-fold decreases in GR protein levels, as well as similar decreases in the level of the GerD germination protein that is required for normal GR-dependent spore germination (26, 28).

Given the relationship between rates of nutrient germination and GR levels in spores, it is of obvious interest to determine the factors that regulate spores' GR levels. The tricistronic operons encoding *B. subtilis* GRs are transcribed by RNA polymerase with the forespore-specific sigma factor σ^G (11, 34). However, there is no knowledge of how σ^G levels may vary between individual spores or how much stochasticity might affect an individual spore's σ^G levels. There is also a forespore-specific transcriptional regulatory protein, SpoVT, which modulates the expression of a number of σ^G-dependent genes (1, 3, 38, 41, 42). SpoVT can be either a positive or a negative regulator of gene expression, and in spores prepared by resuspension in a poor medium, SpoVT represses levels of expression of operons encoding GRs ~3-fold and slightly stimulates the expression of the forespore-specific *spoVA* operon that encodes a number of proteins likely involved in spore germination by allowing the spore's large depot (~20% of spore core dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) to leave the germinating spore, one of the earliest events in spore germination. In contrast to its effects on the expression of the *gerA*, *gerB*, *gerK*, and *spoVA* operons, SpoVT is reported to have a negligible effect on the transcription of the *gerD* gene. As with σ^G, there is no knowledge of if or why SpoVT levels may vary between individual sporulating cells, either by regulatory or by stochastic effects.

Given the significant repressive effects of SpoVT on *gerA*, *gerB*, and *gerK* transcription, with minimal effects on other genes in-

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volved in spore germination, it seemed likely that variation in SpoVT levels would have major effects on spores' GR levels and thus in effect modulate rates of spores' nutrient germination. However, it has been reported that a *spoVT* mutation results in spores that germinate extremely poorly (3). This finding is in contrast to what would have been expected on the basis of knowledge of SpoVT's effects on transcription of operons encoding GRs and effects of GR levels on rates of spores' nutrient germination. Consequently, we have examined the effects of a *spoVT* null mutation on rates of spore germination with both GR-dependent and GR-independent germinants and on the levels of a number of GR subunits, as well as GerD and a SpoVAD protein.

MATERIALS AND METHODS

***B. subtilis* strains used and spore preparation and purification.** The *B. subtilis* strains used in this work were isogenic derivatives of strain PS832, a laboratory *trp*$^+$ derivative of strain 168. Specific strains used were (i) PS533 (33), which is strain PS832 carrying plasmid pUB110 encoding resistance to kanamycin (10 μg/ml); (ii) PS4220 (*spoVT*), which was made in this work by transforming strain PS832 to spectinomycin resistance (Sp^r; 100 μg/ml) with chromosomal DNA from strain IB1 (3, 36) in which the *spoVT* gene has been largely deleted and replaced by a spectinomycin resistance cassette; the presence of the *spoVT* deletion was confirmed by PCR; (iii) FB10 (*gerB**) (23), which carries a point mutation in the *gerBB** cistron such that the resultant GerB GR, termed GerB*, triggers spore germination in response to L-asparagine alone; (iv) PS4222 (*spoVT-lacZ*), which was prepared by transforming strain PS832 to chloramphenicol (5 μg/ml) resistance with chromosomal DNA from strain IB5 (3, 36), which carries a transcriptional *spoVT-lacZ* fusion at the *amyE* locus; and (v) PS4225 (*spoVT gerBB**), which was prepared by transforming strain FB10 to Sp^r with chromosomal DNA from strain IB1; the *spoVT* genotype of this strain was also confirmed by PCR.

Spores of all *B. subtilis* strains were prepared by sporulation on 2× SG medium agar plates without antibiotics or in either rich liquid medium (2× SG) or poor liquid medium (Spizizen's minimal medium without Casamino Acids) as described previously (20, 22, 28, 36). Spores were harvested, purified, and stored in water at 4°C as described previously (20). All spores used in this work were free (>98%) of growing or sporulating cells, germinated spores, and cell debris, as determined by phase-contrast microscopy.

Spore germination and outgrowth. Germination of spores of all strains was preceded by a heat shock (30 min, 70°C), followed by cooling on ice for ≥15 min. Spore germination with the 1:1 chelate of Ca^{2+} and DPA was in 60 mM Ca-DPA at 23°C with spores at an optical density at 600 nm (OD_{600}) of 1.0. Spore germination with Ca-DPA was assessed by phase-contrast microscopy, with ~100 individual spores examined at each time point. Germination of spores with dodecylamine (31) and nutrient germinants was at either 45°C (dodecylamine) or 37°C (nutrient germinants) and with spores at an OD_{600} of 0.5. Routinely, germination was in 200 μl of 25 mM K-HEPES buffer (pH 7.4) plus 50 μM $TbCl_3$, and germination of spore populations was monitored by following DPA release by measurement of Tb-DPA fluorescence in a multiwell fluorescence plate reader as described previously (40, 43, 44, 46). Germinants used were (i) 0.8 mM dodecylamine; (ii) various concentrations of L-valine; (iii) various concentrations of L-asparagine alone; and (iv) various concentrations of L-asparagine plus 10 mM D-glucose, 10 mM D-fructose, and 10 mM KCl (GFK). Rates of spore germination were calculated as described previously (40, 43, 44, 46), and all values shown are averages of results of duplicate measurements on two independent spore preparations. Differences between rates of germination of spores of different strains or spores of the same strain prepared differently were assessed by the two-tailed Student's *t* test.

The germination of multiple individual heat-shocked spores with either 10 mM L-valine or 10 mM all AGFK components as described above was followed by differential interference contrast (DIC) microscopy of spores adhered on a microscope slide as described previously (12, 45, 46). The DIC images of hundreds of individual spores were recorded at a rate of 1 frame per 15 s for up to 120 min, and the image intensities of each individual spore were extracted (45). In these analyses, a spore's DIC image intensity remains relatively constant after mixing with a germinant until a time, T_{lag}, when rapid Ca-DPA release begins. The spore's DIC image intensity then falls rapidly in parallel with Ca-DPA release that ends at $T_{release}$. The parameter $\Delta T_{release}$, which is $T_{release} - T_{lag}$, defines the time for release of ≥90% of a spore's Ca-DPA pool. Following $T_{release}$, there is a further fall of ~30% in a spore's initial DIC image intensity due to hydrolysis of the spore cortex peptidoglycan (PG), with attendant water uptake and swelling of the spore core. The latter process ends at T_{lysis}, with $T_{lysis} - T_{release}$ giving ΔT_{lysis}, the period of cortex hydrolysis and core swelling. Following T_{lysis} there is little or no further change in the spore's DIC image intensity. The values for these kinetic parameters of the germination of individual spores were determined by analysis of between 86 and 271 individual spores that germinated.

The outgrowth of heat-shocked spores was carried out at 37°C in 2× yeast-tryptone (2× YT) medium containing 16 g tryptone–10 g yeast extract–5 g NaCl per liter plus 5 mM L-valine. Spores were added to an OD_{600} of ~0.8, and the OD_{600}s of the cultures were followed over time.

Measurement of levels of GR subunits GerD and SpoVAD. Levels of GR proteins GerD and SpoVAD, both of which are present largely or completely in spores' inner membrane (5, 18, 19, 27, 39), were determined in inner membrane fractions by Western blot analysis using primary rabbit antisera against the various proteins and a secondary antiserum as described previously (7, 13, 14, 28). The primary antisera are specific, and the anti-GR subunit antisera do not cross-react with the analogous GR subunits from heterologous GR subunits (7, 13, 14, 28). In brief, spores were decoated, ruptured by lysozyme digestion, and sonicated briefly to reduce the extract's viscosity and to shear the inner membrane from PG layers, and the inner membrane fraction was isolated by differential centrifugation. Twofold serial dilutions of inner membrane fractions in which levels of germination proteins were to be compared were first run on SDS-polyacrylamide gels and stained with Coomassie blue to determine how much of the different inner membrane fractions were needed to be run on SDS-polyacrylamide gels to load equal amounts of protein. Different amounts of inner membrane protein from different spore preparations were then run together on SDS-polyacrylamide gels, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Billerica, MA), and antigens on the membrane were detected by Western blot analysis using chemiluminescence. Following development of Western blots, the membrane was stripped and then reprobed with another antiserum as described previously (7, 28). Routinely, 8- to 16-fold ranges of inner membrane protein amounts were used to compare levels of germination proteins in different spore preparations, using both visual estimation and the ImageJ program as described previously (7, 28). Differences between levels of various germination proteins in spores of different strains or spores of the same strain prepared differently were assessed by a two-tailed Student's *t* test.

Other methods. Spores to be assayed for β-galactosidase were isolated, decoated, disrupted by lysozyme treatment, and sonicated as described above. After centrifugation in a microcentrifuge, β-galactosidase in the supernatant fluid was assayed fluorometrically using 4-methylumbelliferyl-β-D-galactoside as the substrate and measuring 4-methylumbelliferone as described previously (28). Specific activities of β-galactosidase are expressed in relative fluorescence units (RFU) obtained in a 40-min assay with 10^9 spores. Previous work has shown that DPA levels are essentially identical in spores made in the rich and poor media used in the current work (28). Assays for β-galactosidase were carried out in duplicate on two independent spore preparations, and differences between specific activities in spores prepared differently were analyzed for significance by a two-tailed Student's *t* test. The β-galactosidase specific activity

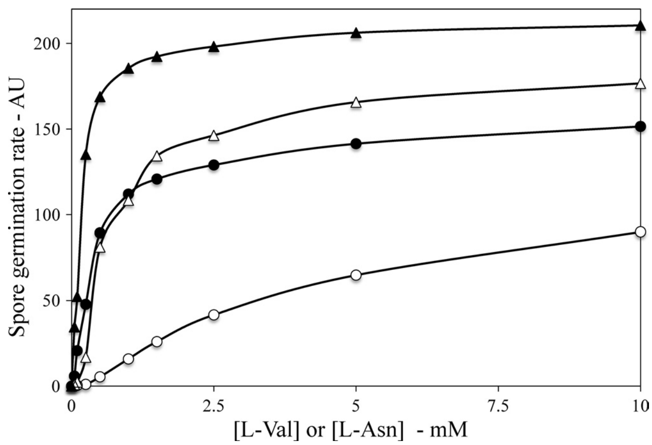

FIG 1 Rates of germination of wild-type and *spoVT* spores with L-valine and AGFK prepared in rich medium. Purified spores of *B. subtilis* strains PS533 (wild type) (○, △) and PS4220 (*spoVT*) (●, ▲) were prepared in a rich liquid medium as described in Materials and Methods. The spores were germinated with various concentrations of L-valine alone (○, ●) or L-asparagine plus GFK (△, ▲), DPA release was monitored to measure spore germination, and spore germination rates in arbitrary units (AU) were determined as described in Materials and Methods. The scales for arbitrary units were the same in all germination experiments. At between 0.05 mM and 10 mM germinant concentrations, differences in rates of germination with L-valine or AGFK between wild-type and *spoVT* spores were highly significant ($P < 0.0001$ to 0.02).

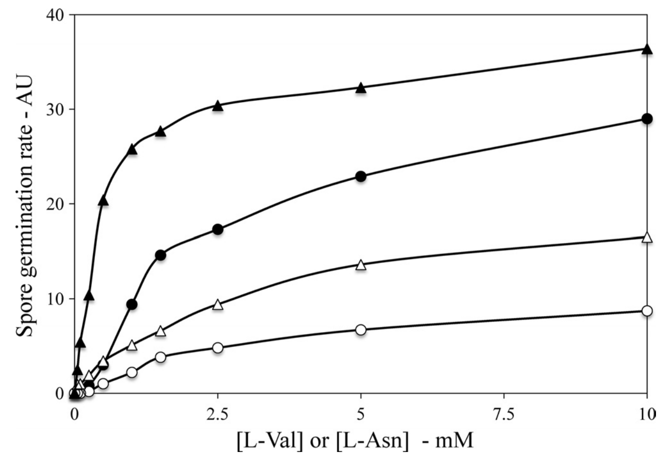

FIG 2 Rates of germination of wild-type and *spoVT* spores with L-valine and AGFK prepared in a poor medium. Purified spores of *B. subtilis* strains PS533 (wild type) (○, △) and PS4220 (*spoVT*) (●, ▲) were prepared in a poor liquid medium as described in Materials and Methods. The spores were germinated with various concentrations of L-valine alone (○, ●) or L-asparagine plus GFK (△, ▲), DPA release was monitored to measure spore germination, and spore germination rates in arbitrary units (AU) were determined as described in Materials and Methods. The scales for arbitrary units were the same as those in Fig. 1. At between 0.25 and 10 mM germinant concentrations, differences in rates of L-valine or AGFK germination between wild-type and *spoVT* spores were highly significant ($P < 0.002$).

of rich-medium spores without a *lacZ* fusion (PS533 spores) was ≤10% of that of PS4222 spores (data not shown).

The level of small, acid-soluble proteins (SASPs) in spores were determined by polyacrylamide gel electrophoresis (PAGE) at low pH of samples from dry ruptured spores (20) and subsequent densitometric analysis of various SASP bands on the stained gel using the program ImageJ. The acetic acid extracts from 6 mg dry ruptured spores were dialyzed, lyophilized, and dissolved in 50 μl of 8 M urea plus 25 μl of acid gel diluent, aliquots were run on polyacrylamide gels at low pH, and gels were stained with Coomassie blue.

The UV resistance of spores of various strains was determined by irradiation at 24°C with a UV lamp with maximum output at 254 nm (UVG-11; UVP, San Gabriel, CA) that was 35 cm from a 35-mm petri dish with 2 ml spores at an OD_{600} of 1.0 in water as described previously (20). Aliquots from unirradiated and irradiated samples were spotted on Luria broth agar plates (22) with the appropriate antibiotic, plates were incubated for 24 to 48 h, and colonies were counted.

RESULTS

Nutrient germination of wild-type and *spoVT* spores. It was reported that spores lacking SpoVT germinate very poorly with nutrients and that SpoVT is a repressor of operons that encode GRs (3, 41). Since GR levels appear to be directly related to spores' rates of nutrient germination (2, 4, 24, 28), these previous results with *spoVT* spores seem somewhat contradictory. One possible explanation for this apparent contradiction could be in the methods used to measure germination, as some methods measure an early event in germination such as DPA release, while others monitor the resumption of metabolism in spores, and this does not take place until both DPA release and cortex PG hydrolysis are complete and spore outgrowth has begun (34, 35). Since the germination of *spoVT* spores in previous work was monitored by measuring the resumption of spore metabolism (3), we examined the nutrient germination of wild-type and *spoVT* spores by monitoring DPA release, one of the earliest measurable events in spore germination (34, 35). In these experiments, spores made in both a rich and a poor liquid medium were examined, since previous work indicated that poor-medium spores germinated relatively poorly and had lower levels of GRs and GerD (28). Perhaps higher levels of SpoVT in developing spores prepared in the poor medium are involved in determining low levels of some germination proteins in poor-medium spores. To examine whether SpoVT levels might be involved in the determination of GR levels in spores made in rich and poor sporulation media, the specific activity of β-galactosidase from a *spoVT-lacZ* fusion was assayed in spores of strain PS4222 (*spoVT-lacZ*) prepared in rich and poor liquid media. Strikingly, the β-galactosidase specific activity in the poor-medium spores (2.1×10^4 RFU/10^9 spores) was twice as high as that in the rich-medium spores (1.1×10^4), and the latter value was ~10-fold higher than the β-galactosidase specific activity in spores of strain PS533, which does not contain a *lacZ* fusion (10^3). The difference in the levels of β-galactosidase in PS4222 spores made in a rich or a poor medium was highly significant ($P <$ 0.0001).

When the levels of germination of *spoVT* and wild-type spores made in either the rich or poor medium were compared, the *spoVT* spores exhibited higher rates of germination with both L-valine via the GerA GR and with the AGFK mixture via the GerB plus GerK GRs (Fig. 1 and 2). The higher rates of germination of the *spoVT* spores were most pronounced at low nutrient germinant concentrations, as has been found previously when rates of germination of spores with elevated GR levels are compared to germination rates of wild-type spores (2, 4). Also as found previously (28), both the wild-type and *spoVT* spores prepared in the poor liquid medium germinated more poorly than rich-medium spores of the same genotype (Fig. 2).

Spores that contained a GerB GR variant, termed GerB*, that can trigger germination with L-asparagine alone and with either

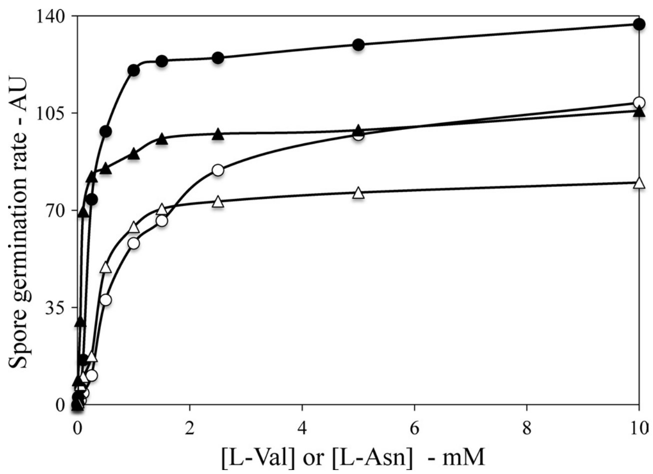

FIG 3 Rates of germination of *gerB** and *gerB* spoVT* spores with L-valine and L-asparagine prepared in a rich medium. Purified spores of *B. subtilis* strains FB10 (*gerB**) (○, △) and PS4225 (*gerB* spoVT*) (●, ▲) were prepared in a rich liquid medium as described in Materials and Methods. The spores were germinated with various concentrations of L-valine alone (○, ●) or L-asparagine alone (△, ▲), DPA release was monitored to measure spore germination, and spore germination rates in arbitrary units (AU) were determined as described in Materials and Methods. The scales for arbitrary units were the same as those in Fig. 1. At between 0.01 and 5 mM germinant concentrations, differences in rates of germination with L-valine or L-asparagine between wild-type and *spoVT* spores were highly significant ($P < 0.001$ to 0.02).

an otherwise wild-type or *spoVT* background were also prepared in a rich medium, and their germination with either L-valine or L-asparagine alone was also measured (Fig. 3). As seen with wild-type and *spoVT* spores prepared in a rich medium, *gerB* spoVT* spores (strain PS4225) germinated much faster than *gerB** spores both with L-valine and with L-asparagine alone, and again, the faster germination of the *gerB* spoVT* spores was most pronounced at lower germinant concentrations.

Kinetic parameters of germination of multiple individual wild-type and *spoVT* spores. During nutrient germination, the release of DPA was clearly faster with *spoVT* spore populations than with wild-type spores. Another way to more precisely determine the differences in the germination of *spoVT* and wild-type spores is to examine kinetic parameters of the germination of multiple individual wild-type and *spoVT* spores. This analysis can determine the average lag time, T_{lag}, between germinant addition and initiation of fast DPA release, the time, $\Delta T_{release}$, for release of ≥90% of a spore's DPA pool, and the time, termed ΔT_{lysis}, for PG cortex hydrolysis and core swelling (12, 40, 46). Consequently, we examined the L-valine and AGFK germination of large numbers of individual wild-type and *spoVT* spores (Fig. 4; Table 1). This analysis showed that T_{lag}s for germination with either AGFK or L-valine were significantly shorter with *spoVT* spores, $\Delta T_{release}$s were essentially identical for both wild-type and *spoVT* spores, and ΔT_{lysis}s were significantly longer for *spoVT* spores (Table 1).

Nonnutrient germination of wild-type and *spoVT* spores. The finding noted above, that *spoVT* spores, as both populations and individuals, germinated significantly faster than wild-type spores, as well as the knowledge that SpoVT represses operons encoding GRs, suggested that the faster nutrient germination of *spoVT* spores might be due to elevated GR levels. However, there are other ways in which spore germination rates can be increased, including alterations in SpoVA protein levels or in spore PG cortex structure (34, 39, 46). To obtain further evidence that the presence of SpoVT in sporulation had specific effects on spore germination via alterations in GR levels, we examined germination of wild-type and *spoVT* spores made in a rich medium with two nonnutrient germinants, dodecylamine and Ca-DPA, neither of which triggers germination via GRs (31, 34) (Fig. 5). As seen with nutrient germination, the germination of *spoVT* spores with dodecylamine was significantly faster than that of wild-type spores (Fig. 5A). However, Ca-DPA germination was significantly slower with *spoVT* spores than with wild-type spores (Fig. 5B). The same results were obtained with two independent sets of spore preparations (data not shown).

Levels of GR subunits, GerD, and SpoVAD in wild-type and *spoVT* spores. The results presented above showed that *spoVT* spores germinated significantly faster than wild-type spores with nutrients and dodecylamine, although not with one germinant, Ca-DPA, which does not trigger germination via GRs. These results plus the reported repression of the *gerA*, *gerB*, and *gerK* operons by SpoVT (3, 41) strongly suggested that *spoVT* spores might have higher GR levels than wild-type spores. To test this suggestion directly, the levels of a number of GR subunits, as well as two additional germination proteins, GerD and SpoVAD, were determined by Western blot analysis of inner membrane proteins from wild-type and *spoVT* spores (Fig. 6; Table 2). Strikingly, levels of the GerAA, GerAC, and GerBC GR subunits were 2- to 8-fold higher in *spoVT* spores, while levels of GerKA and GerD were similar in wild-type and *spoVT* spores and SpoVAD levels were ~2.5-fold lower in *spoVT* spores.

Germination and outgrowth of wild-type and *spoVT* spores. The results noted above indicated that *spoVT* and *gerB* spoVT* spores germinated significantly faster with nutrients than wild-type or *gerB** spores when germination was monitored by measuring DPA release. However, this result does not agree with the results described in a previous report that *spoVT* spores germinate slower than wild-type spores, although in this work, spore germination was followed by measurement of the resumption of spore metabolism (3). One possibility is that while DPA release is indeed faster during germination of spores of *spoVT* strains, perhaps some event later in germination is significantly slower with *spoVT* spores. Indeed, the average T_{lysis}s for *spoVT* spores germinating with either L-valine or AGFK were significantly longer than the T_{lysis}s for wild-type spores (Table 1). To test this suggestion more thoroughly, we measured the ability of wild-type and *spoVT* spores both to initiate germination and to return to active growth by germination of spores in a complete nutrient medium (Fig. 7). In this case, while the germination of the *spoVT* spores, as measured by the fall in the OD_{600} of the spore cultures, was faster than that of the wild-type spores, the return to vegetative growth was significantly slower than that with wild-type spores. This observation suggests that *spoVT* spores are much slower than wild-type spores in either an event in spore germination after DPA release or some early event in spore outgrowth.

UV resistance of and SASP levels in wild-type and *spoVT* spores. The elevated GR subunit levels in *spoVT* spores were consistent with SpoVT acting as a repressor of operons encoding GRs. SpoVT is also reported to affect the transcription of other genes expressed in the developing forespore, in particular, genes encoding the DNA protective α/β-type SASP, although it has minimal effects, if any, on transcription of the gene that encodes spores'

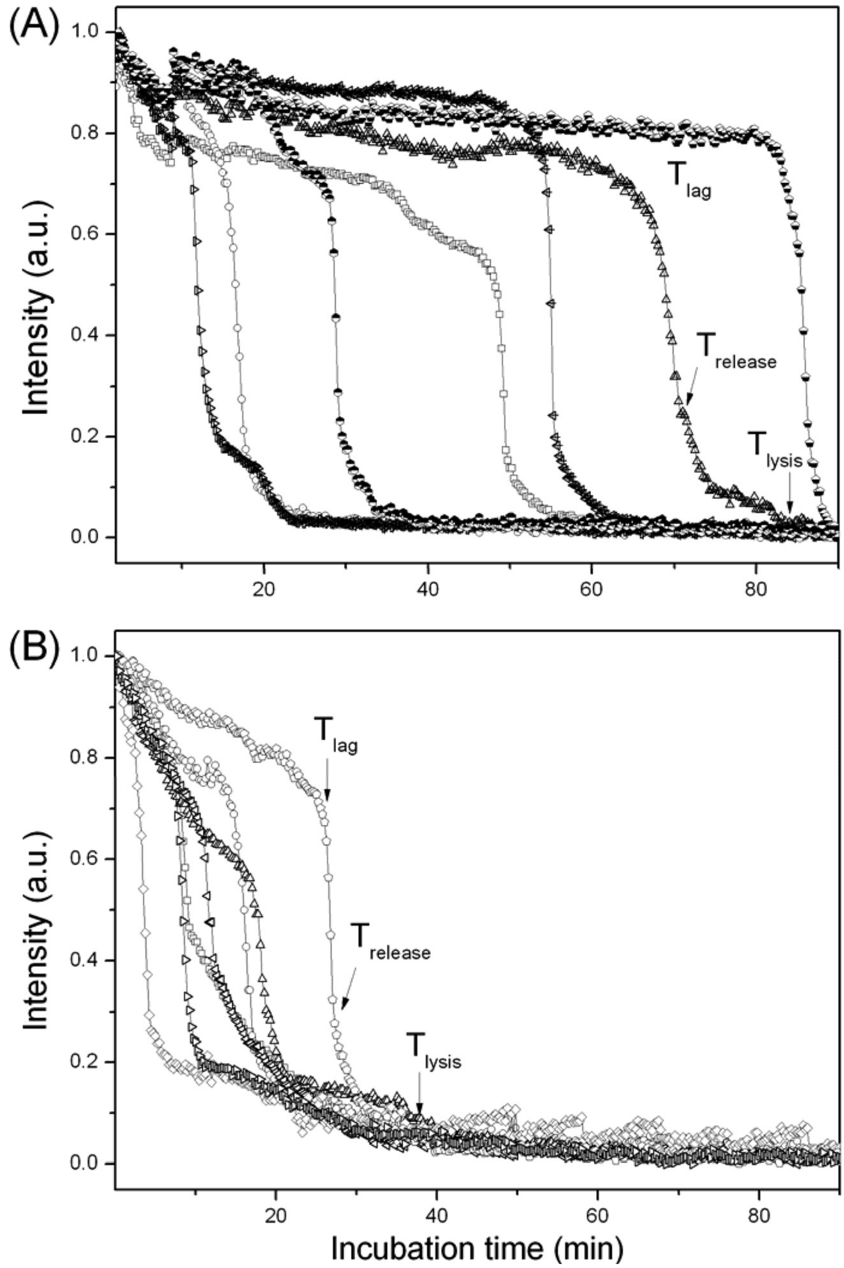

FIG 4 Germination of seven individual wild-type (A) and *spoVT* (B) spores with L-valine. Individual PS533 (wild-type) or PS4220 (*spoVT*) spores were germinated in 10 mM L-valine at 37°C, and DIC image intensities of spores were recorded every 15 s, were normalized to 1.0 at 0 min and to 0.0 after T_{lysis}, and are given in arbitrary units (a.u.). For one spore in each panel, the arrows denote the T_{lag}, $T_{release}$, and T_{lysis} for that spore.

single γ-type SASP, which plays no role in spore DNA protection (35, 41). Since spores with very low levels of SASPs exhibit slower outgrowth than wild-type spores (30, 32), we also examined the levels of SASPs in wild-type and *spoVT* spores (Fig. 8; Table 3). While levels of SASP-γ were essentially identical in wild-type and *spoVT* spores, as expected, levels of the DNA protective α/β-type SASP were ~30% lower in *spoVT* spores when the levels of the α/β-type SASP were expressed relative to the levels of the γ-type SASP (Table 3). Spore UV resistance in particular is very sensitive to spores' level of the α/β-type SASP, with even small decreases in these proteins' levels resulting in significant decreases in spore UV resistance (16). Indeed, *spoVT* spores were more UV sensitive than wild-type spores, consistent with the *spoVT* spores' lower apparent levels of α/β-type SASP, as shown above (Fig. 9).

DISCUSSION

The results reported in this communication provide new information on the reasons for the effects of the SpoVT regulator on the properties of *B. subtilis* spores, as follows. (i) In most cases, the reported effects of SpoVT on transcription of forespore-specific genes were mirrored by the generally similar effects on the levels of a number of spore proteins. Thus, SpoVT is reported to be a repressor of the *gerA*, *gerB*, and *gerK* operons (3, 41), and the levels of all these GRs' subunits except for those of GerKA were elevated

TABLE 1 Germination kinetic parameters of wild-type and *spoVT* spores[a]

Spore	Germinant	T_{lag} (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)	T_{lysis} (min)	ΔT_{lysis} (min)	No. (%) of spores examined
spoVT	L-Valine	6.6 ± 5.7[b]	8.6 ± 6.0	2.5 ± 0.8	25. 9 ± 13.5	17.4 ± 10.5[b]	279 (97.2)
spoVT	AGFK	8.7 ± 7.5[b]	10.9 ± 7.6	2.2 ± 0.7	23.7 ± 10.7	12.8 ± 7.6[b]	99 (86.7)
wt	L-Valine	25.1 ± 25.0[b]	27.2 ± 23.1	2.1 ± 0.7	36.1 ± 23.3	8.9 ± 4.5[b]	138 (84)
wt	AGFK	14.6 ± 11.8[b]	16.9 ± 11.8	2.4 ± 0.6	23.7 ± 11.9	6.8 ± 2.9[b]	117 (94)

[a] The germination of multiple individual spores of strains PS533 (wild type [wt]) and PS4220 (*spoVT*) with either 10 mM L-valine or 10 mM in all AGFK components was measured as described in Materials and Methods. All kinetic germination parameters are shown as averages ± standard deviations.

[b] The differences between T_{lag} and T_{lysis} values for wild-type and *spoVT* spores' germination with either L-valine or AGFK were highly significant ($P < 0.001$).

in *spoVT* spores. The reason that GerKA levels were not affected in the current work are not clear, although in the current work, *spoVT* spores were prepared by nutrient exhaustion in a rich medium, while in the work assessing the effects of SpoVT on forespore-specific gene expression, sporulation was induced by resuspension in a poor medium (3, 41). Levels of SpoVAD and the α/β-type SASP were also lower in *spoVT* spores, consistent with SpoVT's role as an activator of transcription of the *spoVA* operon and at least the *sspB* gene encoding SASP-β, although there is disagreement in the literature on whether SpoVT is also an activator of the *sspA* gene encoding SASP-α (3, 41). Finally, spores' levels of SASP-γ, encoded by the *sspE* gene, and GerD were essentially unaffected by the *spoVT* mutation, consistent with the minimal effects on *gerD* and *sspE* transcription in a *spoVT* strain (3, 41). (ii) Mutants with the *spoVT* mutation are reported to have a coat defect, including a grossly misassembled spore coat, and the spores are slightly lysozyme and chloroform sensitive (3). The *spoVT* spores are also reported to have slightly reduced wet heat resistance, although the spore coat alone does not generally play a significant role in spore wet heat resistance (35). However, *spoVT* spores' more rapid germination with dodecylamine and slower germination with Ca-DPA seen in the current work are similar to the effects of known coat defects on spore germination with these agents (21, 31), consistent with *spoVT* spores having a coat defect. An obvious question is how the action of SpoVT in the forespore affects spore coat assembly. One possibility is that normal coat assembly is dependent on normal forespore development, and there is recent evidence for a role for the forespore in modulating spore coat assembly (17). Another possibility is that it is reduced synthesis of α/β-type SASPs in the forespore that leads to a coat defect, as when α/β-type SASPs are not made in the forespore at levels sufficient to saturate forespore DNA, expression of other forespore-specific genes is altered and there is aberrant expression of at least several coat protein genes as well (32). (iii) The fact that

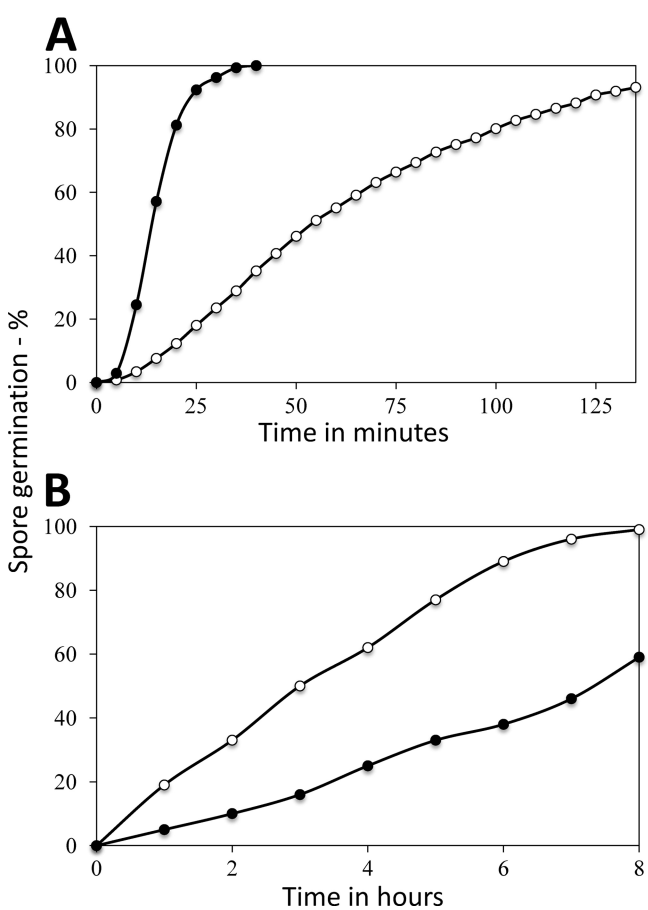

FIG 5 Rates of germination of wild-type and *spoVT* spores with dodecylamine and Ca-DPA. Purified spores of *B. subtilis* strains PS533 (wild type) and PS4220 (*spoVT*) prepared in a rich liquid medium were germinated with dodecylamine (A), for which DPA release was monitored to measure spore germination, and spore germination percentages in arbitrary units (AU) were determined as described in Materials and Methods, or Ca-DPA (B), for which spore germination percentages were determined by phase-contrast microscopy as described in Materials and Methods. Symbols: ○, PS533 (wild-type) spores; ●, PS4220 (*spoVT*) spores.

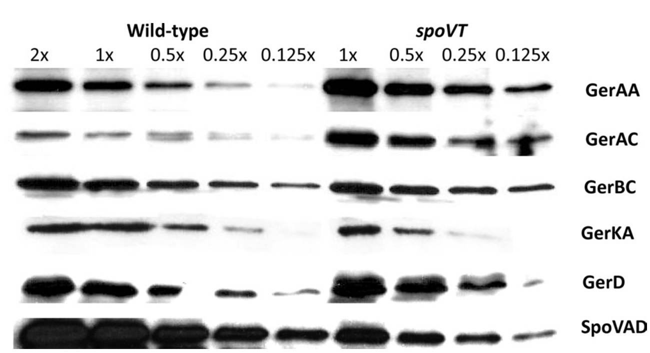

FIG 6 Western blot analysis of levels of germination proteins in wild-type (PS533) and *spoVT* (PS4220) spores. Spores of the two strains were prepared and purified, spores were disrupted, the inner membrane fractions were isolated, and aliquots of various amounts of inner membrane protein were subjected to Western blot analysis as described in Materials and Methods. The amounts of inner membrane protein in the 1× samples from wild-type and *spoVT* spores were identical. The GerAA, GerAC, GerBC, and SpoVAD strips are from the same Western blot that was stripped and reprobed with different antisera. The GerKA strip and GerD strips are each from different Western blots, but ones run with samples from the same spore inner membrane fraction analyzed for GerAA, GerAC, GerBC, and SpoVAD. Note that on the GerD strip there were bubbles on the 0.5× wild-type and 0.125× *spoVT* lanes that eliminated the right halves of these bands.

TABLE 2 Ratios of germination proteins in *spoVT* and wild-type spores[a]

Germination protein	Ratio of protein level in *spoVT* spores/ wild-type spores
GerAA	5
GerAC	8
GerBC	1.8
GerKA	1
GerD	1
SpoVAD	0.4

[a] Spores of strains PS533 (wild type) and PS4220 (*spoVT*) were prepared in rich liquid medium. Inner membrane fractions were isolated from these spores, and relative levels of various germination proteins were determined by Western blot analysis as described in Materials and Methods and in Fig. 6.

spoVT spores have lower levels of α/β-type SASPs was shown here directly and was indicated indirectly by the decreased UV resistance of these spores. Since decreased levels of α/β-type SASPs have been shown to result in decreased spore wet heat resistance (16, 35), this suggests that the decreased wet heat resistance of *spoVT* spores is also due to these spores' low α/β-type SASP levels. It is also possible that SpoVT may modulate the expression of the *spl* gene, involved in repair of specific UV damage in spore DNA, but there are no data available on this point (41).

Other new observations in this work included the finding that the elevated levels of GerAA, GerAC, and GerBC in *spoVT* spores, and thus, presumably, the GerA and GerB GRs, explain at least in part the more rapid GR-dependent DPA release during L-valine and L-asparagine germination of *spoVT* and *gerB* spoVT* spores, especially at low nutrient germinant concentrations (2, 4, 28). The elevated GerBC level in *spoVT* spores is also consistent with the increased AGFK germination seen with spores overexpressing only the GerB GR and not the GerK GR, although there was a much greater increase in the rates of AGFK seen in *spoVT* spores compared with those seen previously when the GerB GR alone was overexpressed ~3-fold (37). The more rapid DPA release from *spoVT* spore populations during nutrient germination appeared to be due to shorter T_{lag}s between nutrient germinant addition and the initiation of rapid DPA release, as the time for rapid release of the great majority of spore DPA during nutrient germination, $\Delta T_{release}$, was essentially identical in wild-type and *spoVT* spores. This is also what has been seen with spores with only a single overexpressed GR when germinated with nutrients that trigger either the overexpressed GerA or GerB* GR (45).

The more rapid GR-dependent germination of *spoVT* spores with L-valine was not unexpected, as noted above. However, while the elevated rates of AGFK and L-asparagine germination of *spoVT* and *gerB* spoVT* spores, respectively, initially appeared to be consistent with the elevated GerBC level in *spoVT* spores, this result was actually quite surprising, because 8-fold higher levels of GerAA and GerAC in wild-type spores actually result in very strong inhibition of AGFK germination via the GerB plus GerK

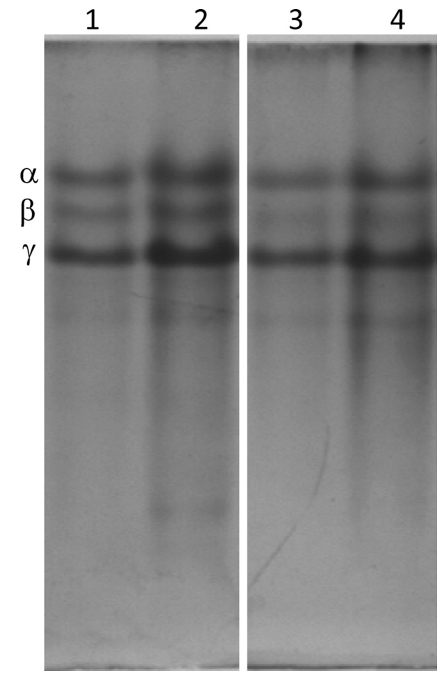

FIG 8 Levels of various SASPs in wild-type and *spoVT* spores. SASPs were extracted from PS533 (wild-type) (lanes 1 and 2) and PS4220 (*spoVT*) (lanes 3 and 4) spores, dialyzed, and lyophilized; aliquots (5 μl, lanes 1 and 3; 10 μl, lanes 2 and 4) of the dissolved lyophilized material were run on polyacrylamide gels at low pH; and the gels were stained with Coomassie blue as described in Materials and Methods. The symbols α, β, and γ adjacent to lane 1 denote the migration positions of SASP-α, -β, and -γ. Note that lanes 1 to 4 were from the same gel, but intervening lanes were removed for clarity.

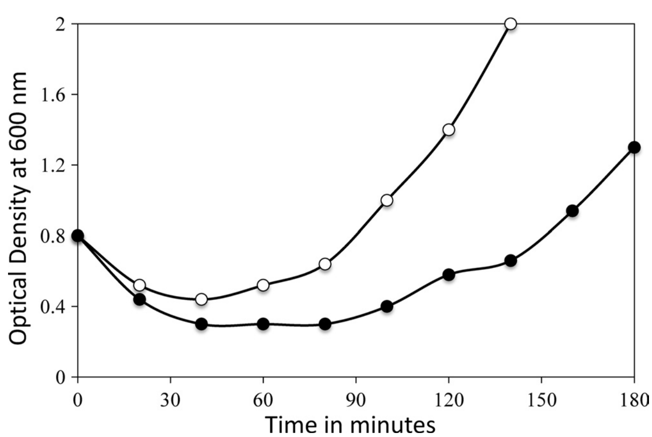

FIG 7 Germination and outgrowth of wild-type and *spoVT* spores. Spores of *B. subtilis* strains PS533 (wild type) and PS4220 (*spoVT*) prepared in a rich liquid medium were heat shocked, cooled, and incubated with shaking at 37°C and an initial OD_{600} of 0.8 in 2× YT medium plus 5 mM L-valine, and the OD_{600} of the cultures was measured over a 3-h period. Symbols: ○, PS533 (wild-type) spores; ●, PS4220 (*spoVT*) spores.

TABLE 3 SASP levels in wild-type and *spoVT* spores[a]

	Level of the following SASP (arbitrary units):			
Spore	α	β	γ	α + β/γ
Wild type	47	31	62	1.3
spoVT	33	19	58	0.9

[a] Spores of strains PS533 (wild type) and PS4220 (*spoVT*) were purified, disrupted, SASP extracted, dialyzed, and lyophilized; the dry powder was redissolved; aliquots were run on polyacrylamide gels at low pH; and gels were stained with Coomassie blue as described in Materials and Methods and shown in Fig. 8. Relative levels of various SASPs were determined by ImageJ analysis of the stained gel shown in Fig. 8. SASP levels are given in arbitrary units, but these units are identical for all SASPs in the spores of both strains.

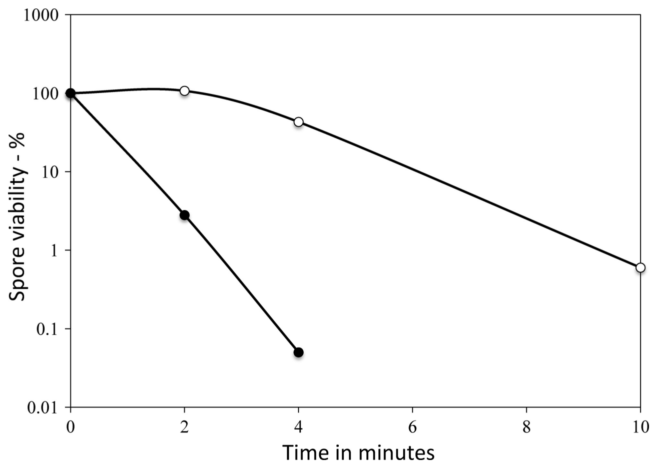

FIG 9 UV resistance of wild-type and *spoVT* spores. Spores of strains PS533 (wild type) and PS4220 (*spoVT*) were exposed to UV radiation, and spore viability was measured as described in Materials and Methods. All values shown are averages of duplicate determinations, and results similar to those shown here were obtained in two separate experiments. Symbols: ○, wild-type spores; ●, *spoVT* spores.

GRs or of L-asparagine germination via the GerB* GR (2, 37). The mechanism whereby the overexpressed GerA GR inhibits germination via other GRs is not known. However, one possible explanation is that all GRs compete for a low-abundance downstream signaling molecule in the spore germination pathway, such that with elevated GerA levels, GerA outcompetes other GRs for access to this signaling molecule, thus inhibiting germination via other GRs. If, however, the gene encoding the downstream signaling molecule is also repressed by SpoVT, then there will likely be more of this molecule in *spoVT* spores, thus allowing more rapid germination with all GRs, even if the GRs are not significantly overexpressed. Unfortunately, while the existence of this downstream signaling molecule has been proposed (2), it has not yet been identified.

The elevated rates of DPA release in GR-dependent germination of *spoVT* spores are in contrast to the report that *spoVT* spores germinate slower than wild-type spores (3). However, clearly, the return to vegetative growth of *spoVT* spores is much slower than that of wild-type spores. The time needed for PG cortex lysis, ΔT_{lysis}, in germination of *spoVT* spores is also longer than that for wild-type spores. The reason for this is not known, but SpoVT is reported to activate *cwlD* expression (41), and thus, *spoVT* cells may have less CwlD during spore formation. Since CwlD is essential for the generation of the cortex-specific modification, muramic acid δ-lactam (MAL) (35), it is possible that the cortical PG in *spoVT* spores has less MAL than that in wild-type spores. If this is the case, even though SpoVT appears to repress expression of one cortex-lytic enzyme (CLE), SleB (42), the degradation of cortex PG might be slower during germination of *spoVT* spores, since MAL is the recognition element for SleB as well as the other redundant CLE, CwlJ (34, 35). Indeed, *spoVT* spores have significantly longer ΔT_{lysis}s for both AGFK and L-valine germination, and SpoVT is also an activator of a spore cortex protein, CoxA (41), although the function of this protein is not known. However, the shorter T_{lag}s for *spoVT* spores make the actual T_{lysis}s for wild-type and *spoVT* spores rather similar, and thus, it seems unlikely that the slow outgrowth of *spoVT* spores is due only to slow cortex hydrolysis. Perhaps the slow outgrowth of *spoVT* spores is due to low levels of some proteins in *spoVT* spores that are needed for rapid spore outgrowth. Indeed, *spoVT* spores have lower levels of α/β-type SASPs than wild-type spores, and low SASP levels alone can slow spore outgrowth even in a rich medium (30), although whether the ~30% lower α/β-type SASP level in *spoVT* spores would cause this effect is not known.

Analysis of the germination of wild-type and *spoVT* spores made in rich and poor media indicated that a *spoVT* mutation increased rates of spore germination 1.5- to 10-fold, depending on the nutrient germinant used and its concentration. Spore levels of β-galactosidase expressed under the control of the *spoVT* promoter were ~2-fold higher in poor-medium spores than in rich-medium spores, suggesting that SpoVT levels are also ~2-fold higher in forespores developing in the poor medium. This is certainly consistent with the slower germination and lower GR levels of poor-medium spores (28), since SpoVT appears to decrease the levels of at least the operons that encode the GerA and GerB GRs. However, a difference in SpoVT level alone in forespores forming in a poor medium is not sufficient to explain the lower germination of poor-medium spores, since poor-medium *spoVT* spores had 2- to 3-fold lower maximal rates of germination than rich-medium *spoVT* spores and even lower rates at subsaturating nutrient germinant concentrations. The identity of other factors that might modulate spore GR levels in a medium-dependent fashion is not known, but a protein phosphatase, PrpE, has been suggested to modulate rates of spore germination and perhaps GRs in some fashion and also modulate SASP levels in spores (9, 15). It may therefore be worthwhile to examine rates of germination and GR levels in spores of a *prpE* strain.

Certainly one of the striking findings from this work is that the SpoVT regulatory protein can have drastic effects on spore properties. These properties include (i) spore germination by modulating the levels of GRs and perhaps other proteins involved directly or indirectly in spore germination and (ii) spore resistance properties by modifying the spore's level of α/β-type SASPs. Since the SpoVT protein appears to be encoded in all Gram-positive spore formers and its amino acid sequence is very highly conserved, these findings suggest that SpoVT levels during sporulation will be an important determinant of levels of at least α/β-type SASPs and germination proteins such as GRs in spores of both the *Bacillales* and *Clostridiales* orders. As a consequence, it may be important to understand the factors that modulate SpoVT expression and levels, as these may be important in modulating both the resistance and the germination of the resultant spores.

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